IS *Presbytis* A DISTINCT MONOPHYLETIC GENUS: INFERENCES FROM MITOCHONDRIAL DNA SEQUENCES

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ABSTRACT

We present a molecular study to examine whether the genus *Presbytis* is monophyletic and distinct from *Trachypithecus*. We sequenced 2300 base pairs of the mitochondrial ND3, ND4L, ND4 and associated tRNAs genes. Five species of *Presbytis* were used including *Presbytis melalophos, P. thomasi, P. comata, P. hosei,* and *P. rubicunda*. *Trachypithecus*, represented by *T. cristatus* and *T. obscurus* and *Nasalis larvatus, Pygathrix nemaues, Colobus guereza, Macaca nemestrina* and *M. fascicularis* were used as outgroups. Our interpretation based on character and distance analyses suggests that *Presbytis* forms its own monophyletic clade distinct from the genus *Trachypithecus*. Relative genetic distance and bootstrap support values from the mtDNA region further confirm the monophyly of *Presbytis*.

Keywords: *Presbytis, Trachypithecus*, mitochondrial DNA, monophyletic, molecular systematics.

INTRODUCTION

At present, very little work has been done on the molecular systematics of Asian colobines. Because of this, Asian colobine systematics has been based on ecological, behavioral and morphological data (Oates *et al*., 1994; Jablonski, 1998; Yan-Zhang *et al*., 1993; Groves, 2001). Of the little molecular work that has been done, most of it has focused on *Trachypithecus* and some of the odd-nosed leaf monkeys (Rosenblum *et al*., 1997; Wang *et al*., 1997; Yaping & Ryder, 1998; Stewart & Disotell, 1998), rather than *Presbytis* itself. Therefore, phylogenetic relationships among the Asian leaf monkeys, particularly *Presbytis* and its relationship to *Trachypithecus*, are not well defined.

Many morphologists and ecologists do not agree on a common delimitation of species within the *Presbytis* group (Groves, 1989; Brandon-Jones, 1995). Formerly, *Semnopithecus* and *Trachypithecus* were grouped into *Presbytis* (Pocock, 1928; Napier, 1985; Wolfheim, 1983). Some Chinese primatologists agree with this arrangement (Peng *et al*., 1988; Li, 1993). Hill (1934) separated these groups from *Presbytis*, at the genus level, and Hooijer (1962) and Eudey (1987) subsequently agree with this assignment. However, Brandon-Jones (1984), Strasser and Delson (1987) and Delson (1994) recognize *Trachypithecus* as the subgenus of the *Semnopithecus*.

The separation of *Trachypithecus* from *Presbytis* has also been adopted by several other researchers (Nowak, 1991; Oates *et al*., 1994; Brandon-Jones *et al*., 2004). However, the variability in the use of the *Presbytis*-*Trachypithecus* clades and their presumed relationship to one another has produced taxonomic and phylogenetic confusion. For this reason, these taxa should be reanalyzed using other systematic approaches such as those provided by molecular analysis. In this study, we examined whether *Presbytis* is a monophyletic group distinct from *Trachypithecus*. This was done by using molecular techniques to determine whether gene sequences found in species of *Presbytis* are phylogenetically distinct from gene sequences found in representative species of the genus *Trachypithecus*, whose members

used to be categorized as members of the genus *Presbytis*.

A robust molecular systematic study should include a phylogenetic analysis of DNA sequences from mitochondrial DNA (mtDNA). Melnick *et al.* (1992) have summarized the uses of mtDNA in primate evolutionary studies. We selected the mitochondrial ND3, ND4L, ND4 genes and three tRNA genes flanking or separating them, because they have been shown in previous studies to resolve Asian primate phylogenetic relationships (Wang *et al.*, 1997; Evans *et al.*, 1999). Using mtDNA gene sequences with its own unique inheritance pattern offers the greatest opportunity to capture the phylogenetic information present in a group of species genetic material.

METHODS

1. Samples

We used five species to represent the genus *Presbytis*: including *P. hosei, P. rubicunda, P. melalophos, P. thomasi* and *P. comata* (Figure 1). Five subspecies of *P. melalophos* were selected, including *P. m. femoralis, P. m. robinsoni, P. m. siamensis, P. m. natunae* and *P. m. mitrata*. We used *T. cristatus* and *T. obscurus* as representatives of the genus *Trachypithecus*. *T. cristatus* has a narrow distribution on the Malay Peninsular and Central Thailand, but is more geographically widespread on Borneo, Sumatra and Indochina (Figure 2). The range of *T. obscurus* is more restricted, extending from the Isthmus of Kra to the Malay Peninsular. We also used *Nasalis larvatus, Pygathrix nemaeus, Colobus guereza, Macaca nemestrina* and *M. fascicularis* as outgroups in order to properly "root" the relationships between the two formerly congeneric groups. Details of genetic samples are in table 1.

2. DNA Sequencing

Total genomic DNA was extracted from tissue or blood using the Qiagen tissue kit with small modifications of standard blood and tissue procedures. We used highly specific primers (T-46PF and T-2409PR), developed by D. T. The, to amplify a segment of mitochondrial DNA spanning the $tRNA^{glyf}$, ND3, $tRNA^{arg}$, ND4L, ND4 and $tRNA^{his}$ genes.

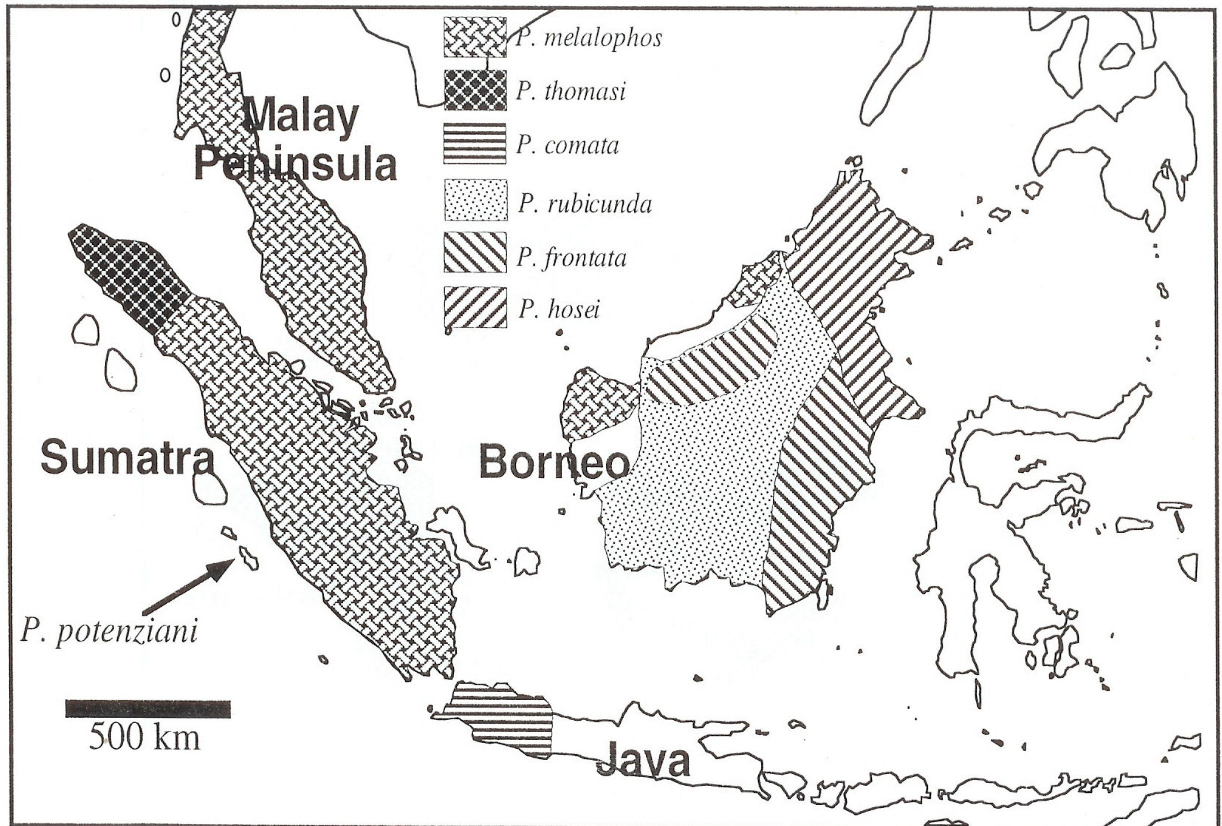

Figure 1. Distribution of species of the genus *Presbytis* (based on Oates *et al.*, 1994).

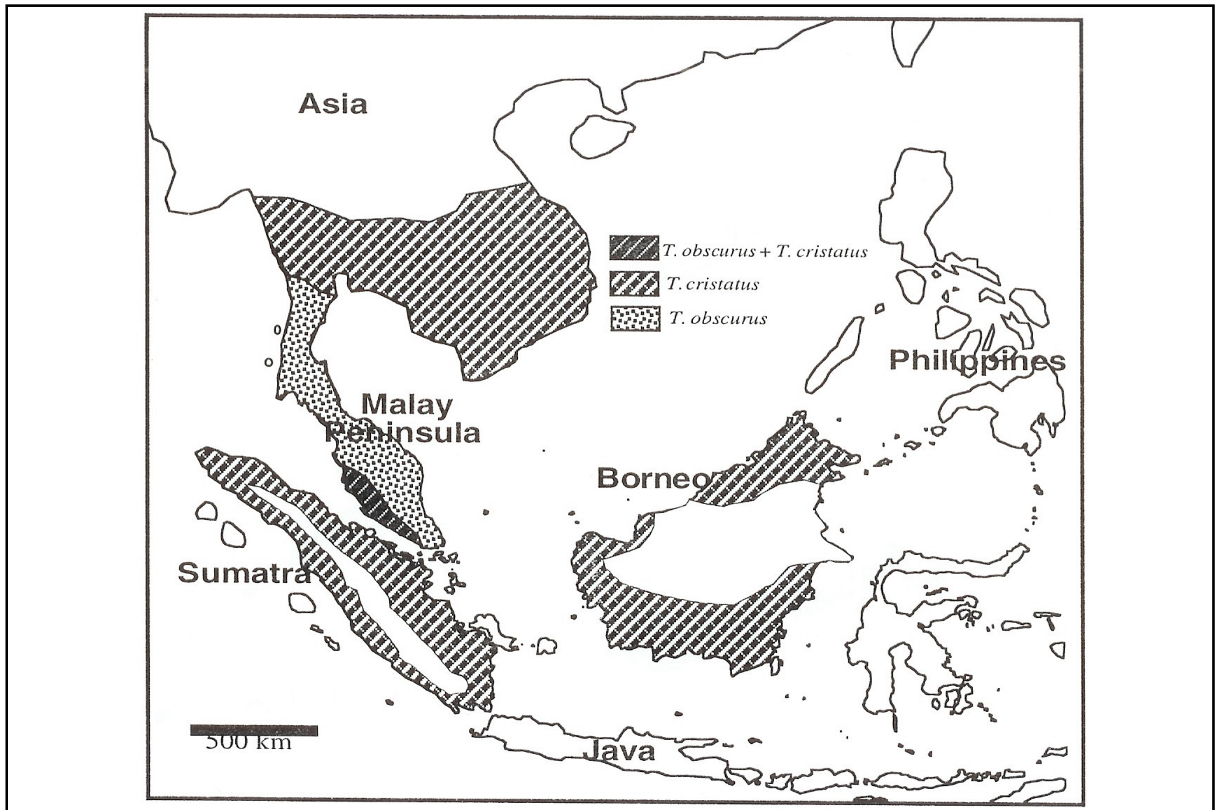

Figure 2. Distribution of the *T. cristatus* and *T. obscurus* (based on Oates *et al*., 1994).

Table 1. Details of genetic samples.

Taxon	Code	Origin
P. melalophos siamensis	BM24	Besut, Terengganu, Malaysia
P. melalophos robinsoni	BM33	Selama, Perak, Malaysia
P. melalophos femoralis	BM36	Kluang, Johor, Malaysia
P. melalophos mitrata	DM4630	Simpai, Sumatra, Indonesia
P. melalophos natunae	DM4609	Natuna Islands, Indonesia
P. rubicunda	Tawau	Tawau, Sabah, Malaysia
P. thomasi	DJ4626	North Sumatra, Indonesia
P. comata	DJ4572	West Java, Indonesia
P. hosei	BM67	Tawau, Sabah, Malaysia
T. cristatus	BM1B	Kuala Selangor, Malaysia
T. cristatus	BM1A	Kota Kuala Muda, Malaysia
T. obscurus	BM8	Sik, Kedah, Malaysia
T. obscurus	BM4B	Taiping, Perak, Malaysia
T. obscurus	BM5B	Kota Kuala Muda, Malaysia
N. larvatus	BM91	Bintagor, Sarawak, Malaysia
N. larvatus	BM93	Kuching, Sarawak, Malaysia
N. larvatus	BM94	Simunjan, Sarawak, Malaysia
Py. nemaeus	DJ9018	Cuc Puong Center, Vietnam
Py. nemaeus	No.2.the	Quang Nam, Vietnam
C. guereza	Cg	Kenya, Africa
M. fascicularis	DM9042	Hanoi, Vietnam
M. nemestrina	BM96	Kuching, Sarawak, Malaysia

Table 2. Oligonucleotide primer pair used in this study and their PCR conditions.

ND3, ND4L, ND4,tRNAs	Forward/Reverse Primer Sequences T-46PF (5'- CTT CCA ATT AGC TAG TTT CGA TA-3') T-2409PR (5'-GCA TGG ATT AGC AGT CCT TGC AAG CT-3')
PCR conditions	Thermocycling parameters were 35 cycles of denaturing at 94°C (1 min), annealing at 56°C (1 min) and extension for 3 min at 72°C.

We carried out 50µl amplifications in a Perkin Elmer Model 480 thermal cycler. A sample of DNA was subjected to 35 cycles of amplification. Each PCR reaction contained 1.0 units of Taq DNA polymerase (Perkin Elmer), 20 pm/µl of each primer, 1µl of dNTPs, 8µl of Buffer A, and 0.5µl of DMSO. Table 2 lists conditions that were used to successfully amplify genes mtDNA region. We loaded our PCR products onto 1.5% agarose gels for electrophoresis. When amplifying the mitochondrial genome, we took precautions to reduce the possibility that our analysis would be affected by nuclear insertions of mtDNA pseudogenes. To do this, we followed the methods of Morales & Melnick (1998). First, our initial amplifications were of very long segments (>2kb). Second, we ran our PCR products in agarose gels and made sure that there was only a single bands we cut out the correctly sized band, which was consistently the strongest amplification product before conducting subsequent amplifications or sequencing. Finally, our results from these steps resulted in mtDNA sequences congruent with the other studies of some of the same taxa for the same region (Wang *et al*., 1997; Evans *et al*., 1999).

Final PCR products were cleaned using the Qiagen PCR Purification Kit and made ready to proceed with cycle sequencing. We performed cycle sequencing with sets of internal primers (Table 3) and the Big-Dye sequencing kit (Perkin Elmer) using the protocols supplied by the manufacturers with the modification that all reaction volumes equal 9µl. We cleaned sequencing products of excess dyes with CentriSep Spin Columns (Princeton separations). We electrophoresed the sequencing products on a 4.25% polyacrylamide gel (19:1 Acryl/Bis gel stock, AMRESCO) and scored the results on an ABI 377 PRISM automated DNA sequencer (Perkin Elmer).

Table 3. List of internal primer sequences.

ND3-ND4	Sequence (5' to 3')
GLYF	ACT TCC AAT TAG CTA GTT T
ND4#1	CTT CTA ACA CTR ACC GCC TGA CT
NAP2M	GGA GCT TCA ACG TGG GCT TT
ARGREV2	TAG ATT ART ATG CCT AGG AGT G
ND4LM	CTA ATA TGC YTA GAA GGA ATA AT
ND4SREV2	AAG AAT TAT TTT TAG CAT TG
NEWND4M	AAT ACC CCT ATA TGG YCT ACA CCT ATG
LEAF1	CCC TGA AGC TTY ACT GGC GCT AT
FORMREVLPRIM2	CTT CAR AAG GCT ATT AGT GG
	TAC ATG TAC ATT ACA ACC CAA CGA GG
T-577F	CTC ACT CCT GGG CAT ATT
T-729F	CTC ACT CCT GGG CAT ATT
T-1089F	CAG CAG TAG GCC TTG C
T-1465F	AAA GCC CAT GTT GAA GC
T-1731F	TGA AGC TTT ACT GGC GC
T-1798R	CGG CTG TGG GTT CGT TC
T-1210R	GCG TTG AGG CGT TCT GCT TG
T-827R	TGG AAA ATC ATG TTG TTG GT
T-304R	GTT GTT TGG AGG GCT CAT GG

3. Analyses

We identified the ambiguous flanking regions of each sequence and removed them from the data using the FACTURA program (ABI, Perkin Elmer). We overlaid all sequences of a particular gene using the AutoAssembler software and then aligned them by eye against the homologous regions in the human mtDNA and nuclear genomes (e.g. Anderson *et al*., 1981).

We analyzed our data using two primary methods to discern phylogenetic relationships: maximum parsimony (MP) and neighbor joining (NJ). These analyses were conducted using PAUP version 4.0 (Swofford, 1999). For unweighted MP, we obtained trees by heuristic searches treating all nucleotide substitutions as unordered. Heuristic searches used random addition of sequences with ten replications. Our data were also subjected to bootstrap analysis with 2000 replications to assess the strength of support for any particular clade (Felsenstein, 1985). We further analyzed the mtDNA data using a weighted MP in accordance with the proportions (3:8:1) we calculated from transition and transversion (TI/TV) ratios in the first, second and third codon position using MacClade 3.0 (Maddison & Maddison, 1992). We also constructed trees using the NJ method by employing the Tajima and Nei distance option of PAUP. We quantified homoplasy using the consistency index (CI) and the homoplasy index (HI). We calculated TI/TV ratios using PAUP based on pairwise base differences.

RESULTS

1. Sequence Variation

We excluded the sequences of $tRNAg^{lyf}$ and $tRNA^{his}$, since these regions contained only a small highly conserved part of the fragment analyzed and only partial $tRNAg^{lyf}$ and $tRNA^{his}$ gene sequences were obtained. The complete DNA sequence for the ND3, $tRNA^{arg}$, ND4L and ND4 contains 2080 base pairs. We combined sequences from these regions because these four loci are tightly linked to each other and the combination gave better phylogenetic resolution (Wang *et al*., 1997). The following analyses are based on approximately 2.1Kb of DNA sequence.

We plotted numbers of transitional and transversional changes against uncorrected p-distances using Microsoft Excel. Our graphs show a linear relationship (Figure 3) implying that no saturation has occurred in these sequences. Table 4 compiles data on TI/TV ratios. Our results indicate that region of ND3, $tRNA^{arg}$, ND4L and ND4 possess high ratio of TI/TV (5.67:1) as compared to nuclear regions (Md-Zain, 2001). Since our data sets include *Presbytis, Trachypithecus, Nasalis, Pygathrix, Colobus* and *Macaca*, these results indicate that the mtDNA region evolves more rapidly in these six genera compared to the autosomal and Y-chromosome regions (Md. Zain, 2001).

2. Phylogenetic Resolution

Figure 4 and Figure 5 show the tree topologies obtained from unweighted MP and NJ analyses of the complete mtDNA dataset. These two topologies are remarkably congruent with respect to the phylogenetic position of the *Presbytis* genus, its member species, and the outgroups *Trachypithecus, Nasalis* and *Pygathrix*. Unweighted MP analysis produced a single bootstrap tree (length=1773, CI=0.5324, HI=0.4676) with 100% bootstrap support for a single clade containing *P. hosei, P. rubicunda, P. comata. P. thomasi* and *P. melalophos*. *T. cristatus* and *T. obscurus* formed a separate monophyletic *Trachypithecus* clade, also with 100% bootstrap support. Weighted MP analysis (tree not shown) also supports the tree topology from unweighted MP in terms of the monophyletic position of the *Presbytis* species. Finally, *Nasalis* and *Pygathrix* also sorted to distinct monophyletic clades each with 100% bootstrap support. The NJ tree topology is somewhat more resolved than the MP tree, but it still supports a distinct monophyletic clade for the *Presbytis* species. We did not employed maximum likelihood analysis as NJ and MP analyses have already portrayed the distinct monophyletic clades with high bootstrap support.

DISCUSSION

We have generated tree topologies from mtDNA region using character state and distance methods of analysis. All tree topologies agree that *Presbytis* species form a single

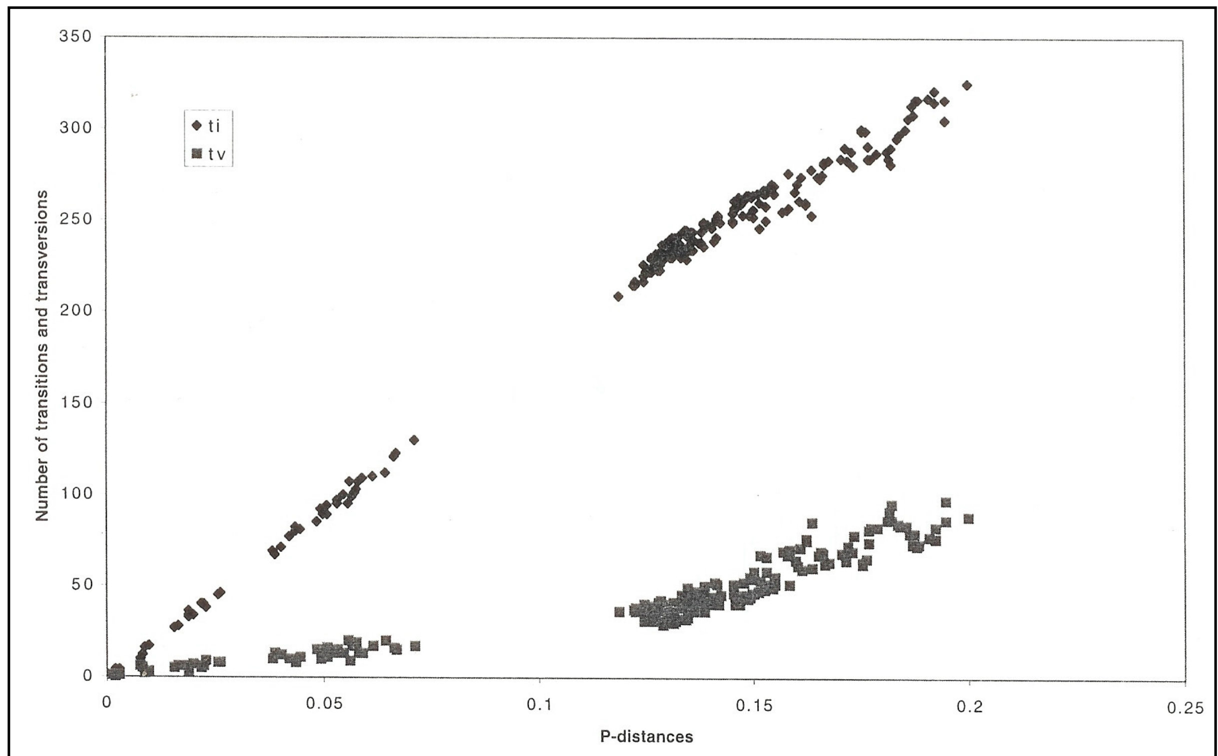

Figure 3. Plot of number of transitions and transversions vs. pairwise uncorrected p-distance from mtDNA data set.

monophyletic clade, distinct from the genus *Trachypithecus*. This distinction is strongly supported by 100% bootstrap values. Therefore, we argue these results strongly support the taxonomic arrangement of Oates *et al*. (1994), Groves (2001) and Brandon-Jones *et al*. (2004) in which *Presbytis* species are separated from *Trachypithecus* species with each being placed in their own distinct genus.

Besides looking at tree topologies and bootstrap values, support for a distinct monophyletic relationship among *Presbytis* species can also be derived from the Tajima and Nei distance matrix. Table 5 summarizes the average percentage sequence divergence values calculated using Tajima and Nei's algorithm (Tajima & Nei, 1984) among *Presbytis* species, *Trachypithecus* species, *Nasalis* and *Pygathrix*, as well as between these groups. The average intra-generic genetic distance ranges from 4.50% in the Presbytis genus to 4.63% in the *Trachypithecus* genus. The average genetic distance between *Presbytis* and *Trachypithecus* species is 15.17%.

These results, while not conclusive, show that intergeneric differences between *Presbytis* and *Trachypithecus* are much greater than the interspecific differences in either genus: three

Table 4. Summary of variations along the sequences across taxa[a].

	mtDNA
Total characters	2080
Constant characters	1276
Parsimony-uninformative characters	154
Parsimony-informative characters	650
% informative No. characters	31.25
Ratio TI/TV from pair wise base differences (PAUP)	5.67
Tree length	1773

[a] All gaps were excluded from analyses. The numbers of unambiguous transitions and transversions were generated from pairwise base differences using PAUP.

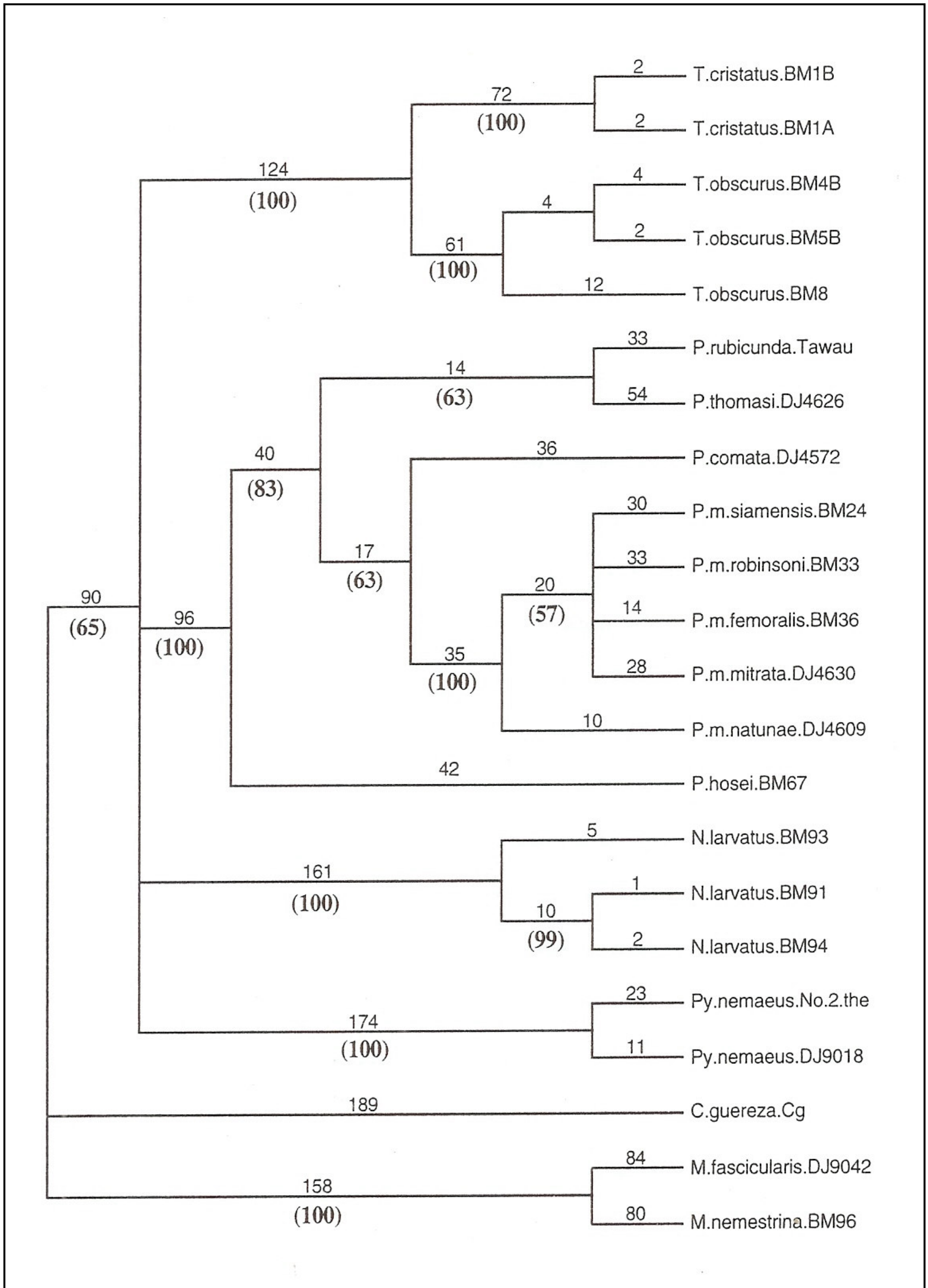

Figure 4. The maximum parsimony heuristic bootstrap tree. The bootstrap support values are shown below the branches of the parsimony tree.

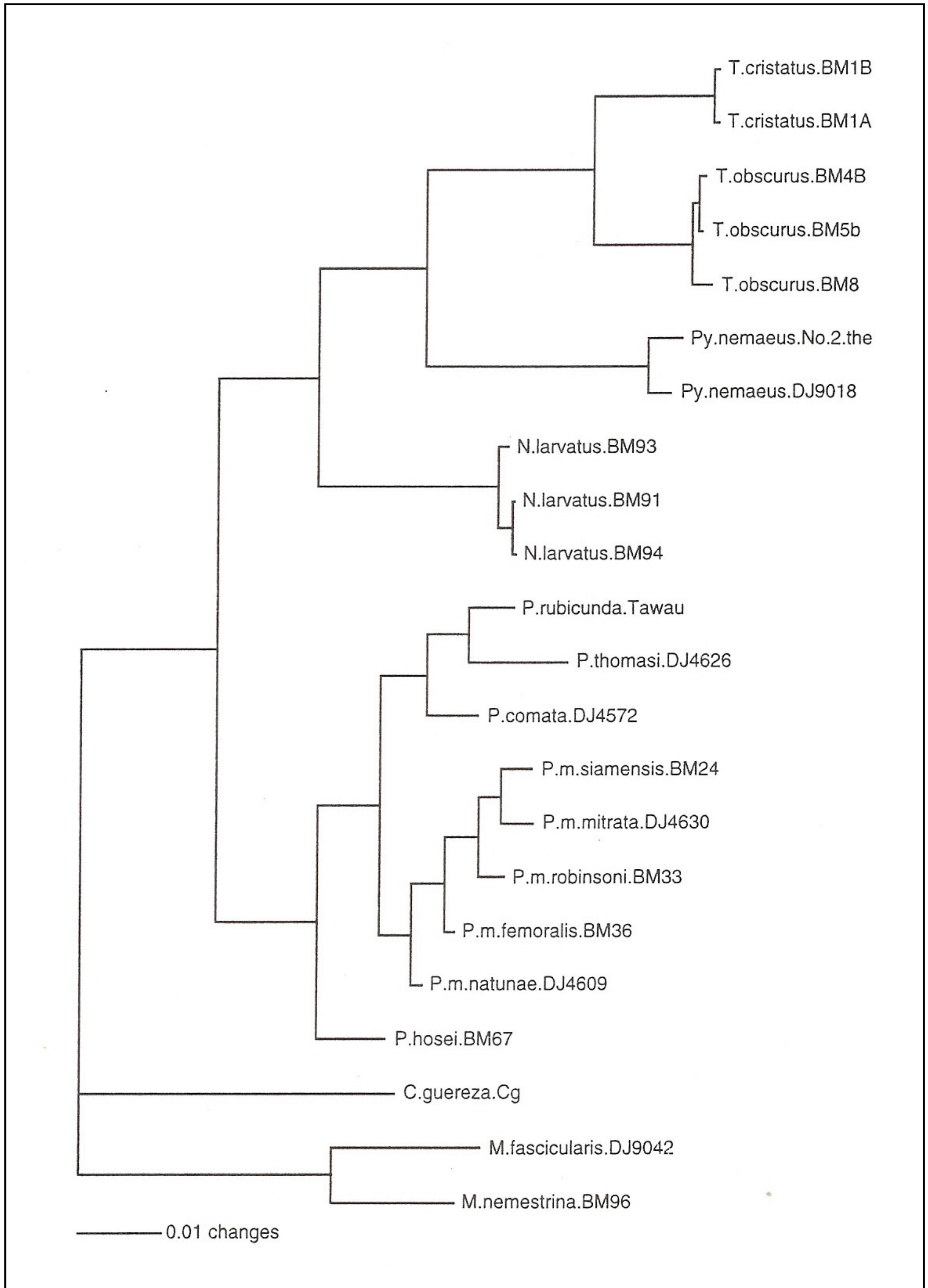

Figure 5. The neighbor-joining tree.

Table 5. Average percentage of genetic distance among and between *Presbytis, Trachypithecus, Nasalis*, and *Pygathrix* using the Tajima and Nei distance.

mtDNA	*Presbytis*	*Trachypithecus*	*Nasalis*	*Pygathrix*
Presbytis	4.496			
Trachypithecus	15.170	4.626		
Nasalis	14.790	16.770	-	
Pygathrix	16.571	18.087	18.378	-

times for mtDNA region. Data clearly show considerably greater genetic divergence between *Trachypithecus* and *Presbytis* than within either clade. Therefore, we strongly argue that these phenetic differences further validate the phylogenetic separation of *Trachypithecus* and *Presbytis* into two separate genera. In addition, the genetic distance values we obtain, for example between genus *Trachypithecus* and *Pygathrix* (18.1%), are in the range of those found by Wang *et al*. (1997) (e.g. 17.6%) using the same genes (ND3-ND4). Similarly, Rosenblum *et al*. (1997) found a genetic distance between *P. comata* and *T. cristatus* of 20.4%, while we found a distance of 15.5% between these same species at the same loci. These latter estimates are probably not significantly different given Rosenblum *et al*. (1997) used restriction site data as opposed to DNA sequence data to estimate genetic sequence divergence. Thus, we gain further confidence that our estimates of between-genus sequence divergence are accurate and the separation of *Presbytis* species and *Trachypithecus* species into two separate genera is well supported.

CONCLUSION

Frequently, *Semnopithecus* and *Trachypithecus* have been grouped with *Presbytis* (Groves, 1970; Wolfheim, 1983) as one large heterogeneous genus. Some primatologists agree with this arrangement (Peng *et al*., 1988; Li, 1993). However, Hill (1934) and Hooijer (1962) subdivided the genus *Presbytis* by elevating the subgenera *Semnopithecus* and *Trachypithecus*, to the generic level and retaining *Presbytis* for a distinct subset of species. Ecological, behavioral, and morphological data clearly support the separation of *Trachypithecus* from *Presbytis* (Hooijer, 1962; Weitzel & Groves, 1985; Oates *et al*., 1994; Nowak, 1991; Groves, 2001; Brandon-Jones *et al*., 2004). Our molecular data have further corroborated this taxonomic distinction. DNA sequence data from mitochondrial region have distinguished *Trachypithecus* from *Presbytis*. Tree topologies from different kinds of phylogenetic analyses clearly indicate that *Presbtyis* and *Trachypithecus* form their own distinct monophyletic clades. Bootstrap values strongly support the topologies obtained, which in turn support the phylogenetic hypothesis of two separate genera. Genetic distance patterns are also congruent with these results.

The weight of all evidence strongly supports the separation of *Presbytis* and *Trachypithecus* into two separate clades, and possibly genera. What now needs to be done is further work to define the molecular phylogenetic position of *Semnopithecus* with respect to the distinct *Presbytis* and *Trachypithecus* clades. Since *Semnopithecus* has been previously grouped with *Presbytis* (Pocock, 1928; Groves, 1970; Wolfheim, 1983) and some primatologists have placed *Trachypithecus* as a subgenus of *Semnopithecus* (Strasser & Delson, 1987; Delson, 1994; Brandon-Jones, 1996), it is of considerable interest as to where *Semnopithecus* fits in relation to these other Asian colobine genera. We, therefore, suggest that further molecular analysis be done to resolve this important, related phylogenetic issue.

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